# Purine- and Nucleotide-mediated Relaxation of Rabbit Thoracic Aorta: Common and Different Sites of Action

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**Abstract**—The mechanisms of the relaxant effect of purines and pyrimidines in New Zealand rabbit isolated aorta were investigated at endothelial and smooth muscle cell levels. Endothelium-mediated relaxation by ATP was only partially inhibited by the $P_2$-purinoceptor antagonist suramin (0·1 mM). The pyrimidine UTP produced vasodilation by acting at the endothelial level and relaxation was not antagonized by suramin (0·1 mM). This effect was not mediated by $P_2$ purinoceptors, indicating that UTP, like ATP to a certain extent, produces relaxation via an endothelium nucleotide (N) pyrimidinoceptor. ATP, ADP, AMP, adenosine, 5'-$N$-ethylcarboxamidoadenosine (NECA) and inosine were all active as relaxants on smooth muscle. The NECA relaxant effect was not antagonized by $P_1$-purinoceptor antagonists 3,7-dimethyl-1-propargylxanthine (50 $\mu$M) or 1,3-dipropyl-8-(2-amino-4-chlorophenyl)xanthine (5 $\mu$M), excluding a $P_1$-mediated effect. $P_2$-related activity was excluded because adenosine-mediated relaxation was not antagonized by suramin (0·1 mM). UTP was ineffective as a relaxant at smooth muscle level, thus excluding the presence of muscular nucleotide (N) pyrimidinoceptor and suggesting a $P_3$ purinoceptor. The rank order of potency of this muscle purinoceptor was NECA > adenosine > ATP ≅ ADP ≅ AMP ≅ inosine.

Adenine nucleotides produce a large variety of physiological responses in many tissues and cell types. The functional effects elicited in the vascular wall consist of vasoconstriction and vasodilation, due to activation of different extracellular receptors. They were first described by Burnstock (1978) as $P_{2x}$ and $P_{2y}$ purinoceptors, mediating contraction and relaxation of vascular smooth muscle, respectively. Since then, many other sites of ATP action have been described according to different agonist rank order of potency. Regarding the relaxant effect, atypical $P_2$ purinoceptors have been proposed, linked to phospholipase C-activation (Allsup & Boarder 1990; Tada et al 1992; Wilkinson et al 1993) or prostacyclin release (Needham et al 1987; Demolle et al 1988). Some of these purinoceptors have been tentatively grouped as nucleotide receptors (Davidson et al 1990; O'Connor et al 1991) and are activated with similar potencies by UTP and ATP. The mechanism of action of the nucleotide receptor has been found to involve activation of phospholipase C (O'Connor et al 1991). However, further characterization is made difficult by the lack of specific agonists and antagonists.

The non-$P_2$ and non-$P_1$ muscular-relaxant effect of ATP in rabbit aorta has recently been attributed to a $P_3$ purinoceptor which is activated by adenosine and ATP (Chinellato et al 1992b). In the present study, we investigated the possibility that the proposed nucleotide and $P_3$ purinoceptor could be identified as the same receptor. Its location in the endothelial layer besides smooth muscle was also considered, and different purine and pyrimidine compounds were tested to characterize the spectrum of receptor-activating drugs.

## Materials and Methods

Experiments were carried out on New Zealand rabbits of both sexes (3 months of age, 2–2·5 kg body weight). The

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rabbits were fed a standard cholesterol-free 2RB15-GLP diet (Mucedola, Milan, Italy) and water was freely available.

After deep anaesthesia with pentobarbitone (25 mg kg$^{-1}$, i.v.), the thoracic aorta was removed and cleaned of adjacent tissue. Special care was taken to avoid contact with the luminal surface to preserve the endothelium. At some points the endothelium was deliberately removed by gently rubbing the intimal surface of the vessel with a pipe cleaner. The descending thoracic aorta was cut into transverse rings approximately 3 mm thick. Two consecutive rings were tied together with silk thread, thus creating a 2-ring chain, in order to increase the mechanical response and the signal/noise ratio. The preparation was suspended in a 30-mL tissue-bath containing modified Krebs-bicarbonate solution of the following composition (mM): NaCl 116·0; KCl 3·2; $CaCl_2$ 1·2; $MgCl_2$ 1·2; $NaH_2PO_4$ 1·2; $NaHCO_3$ 22·0; glucose 10·1; ascorbic acid 1·1, and equilibrated with a 95% $O_2$–5% $CO_2$ gas mixture, pH 7·4, at 37°C.

Isometric tension was recorded by means of force transducers (Type DY0 Basile, Comerio, Italy) connected to a chart recorder (Unirecord Basile, Comerio, Italy). The aortic chains were held at a resting tension of 35 mN and allowed to equilibrate at optimal length for 60–90 min before experiments were started, the buffer being changed every 15 min.

### Experimental protocol

All aorta preparations were tested with the appropriate EC50 of noradrenaline (0·3 $\mu$M), washed and equilibrated for at least 60 min before experiments were started. This procedure was found to increase and stabilize any subsequent contractile response to noradrenaline.

One aorta preparation was used to determine the cumulative concentration-response curve of noradrenaline in order to calculate the EC50 of the contractile agonist for each rabbit. For relaxation studies, vasodilator drugs were cumulatively added to aorta rings precontracted with EC50 noradrenaline to steady-state tension. The drugs were separ-

ately tested at randomly selected tracts of thoracic aorta from each animal.

At the end of the experiments, EC50 noradrenaline was added to verify the stability of the contraction. The spontaneous relaxation following this contraction was subtracted from the relaxation caused by the vasodilator agonists in the previous curve, to calculate the net effect of the agonists. In aorta preparations without endothelium a final maximal relaxant acetylcholine concentration (3 μM) was added to verify correct de-endothelialization.

## Drugs and reagents

Adenosine 5'-triphosphate (ATP) sodium salt, adenosine 5'-diphosphate (ADP) sodium salt, adenosine 5'-monophosphate (AMP) sodium salt, adenosine, inosine, uridine 5'-triphosphate (UTP) and noradrenaline bitartrate were purchased from Sigma Chemical Co. (St Louis, MO, USA). 3,7-Dimethyl-1-propargylxanthine (DMPX), 1,3-dipropyl-8-(2-amino-4-chlorophenyl)xanthine (PACPX) and 5'-N-ethylcarboxamidoadenosine (NECA) were purchased from RBI Research Biochemicals Inc. (Natick, MA, USA). Suramin was obtained as a generous gift from Bayer AG.

NECA, DMPX and PACPX were dissolved in ethanol. All other drugs were dissolved daily in saline. Samples were added to the tissue-bath fluid to obtain the final concentration desired.

## Statistical analysis

The percentage of relaxation by each drug was calculated by taking maximal contraction at steady state induced by noradrenaline EC50 as 100%. Data were expressed as means ± s.e. Differences between means were compared by Student's two-tailed $t$-test for unpaired data, and $P < 0.05$ was accepted as significant.

## Results

### Relaxation induced by adenine nucleotides and adenosine

Fig. 1A shows concentration-response curves of ATP in aorta with or without endothelium, precontracted with EC50 noradrenaline. Relaxation was obtained with concentrations ranging from 30 μM to 10 mM. Mechanical de-endothelialization significantly impaired relaxation, but the maximal effect was reached in both conditions.

ADP induced concentration-dependent relaxation in aorta rings both with and without endothelium (Fig. 1B) with concentrations ranging from 3 μM to 1 mM. The relaxing effect was significantly reduced in rings mechanically deprived of endothelium.

Fig. 1(C, D) show the relaxant effect of AMP (30 μM–1 mM) and adenosine (1 μM–1 mM) in thoracic aorta in the presence and absence of endothelium. No significant difference was detected between the relaxation observed with and without endothelium.

### Inhibition of ATP-induced relaxation by suramin

Fig. 2A shows the relaxant effect of ATP in intact aorta preparations, that is, with endothelium, in the presence or absence of suramin (0·1 mM), preincubated for 20 min before the cumulative addition of ATP. Suramin antagonized ATP relaxation, the difference being significant at concentrations
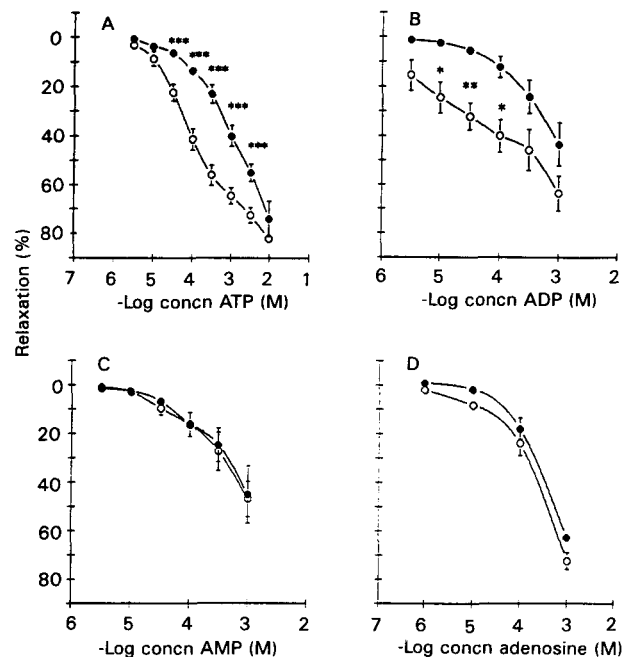


Fig. 1. Response of thoracic aorta to ATP (A), ADP (B), AMP (C) and adenosine (D) in the presence (O) or absence (●) of endothelium. Each point is the mean value ± s.e. from 5–9 rabbits. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
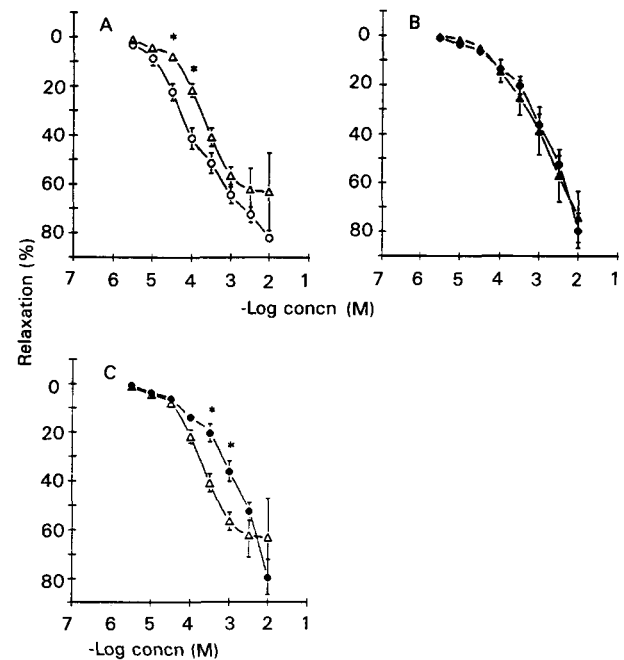


Fig. 2. A. Relaxing response to ATP of thoracic aorta, with intact endothelium, in the presence (△) or absence (O) of suramin (0·1 mM). B. Relaxing response to ATP of thoracic aorta, deprived of endothelium, in the presence (▲) or absence (●) of suramin (0·1 mM). C. Relaxing response to ATP of thoracic aorta, deprived of endothelium (●), or in vessels with intact endothelium, in the presence of suramin (0·1 mM) (△). Each point is the mean value ± s.e. from five rabbits. *$P < 0.05$.

of 30 and 100 $\mu$M. In the absence of endothelium, suramin (0·1 mM) did not affect ATP relaxant activity (Fig. 2B). The overlap of the curve for the muscular effect of ATP and that in the presence of endothelium after preincubation with suramin shows a significant difference, as the relaxant response of intact aorta in the presence of suramin is higher (Fig. 2C).

### Suramin effect on UTP-induced relaxation

UTP induced vascular relaxation in intact aorta preparations in the presence or absence of suramin (0·1 mM) (Fig. 3A). After de-endothelialization, the relaxant effect was significantly reduced and almost abolished at the concentration range tested (1 $\mu$M–1 mM). In intact vessels preincubated
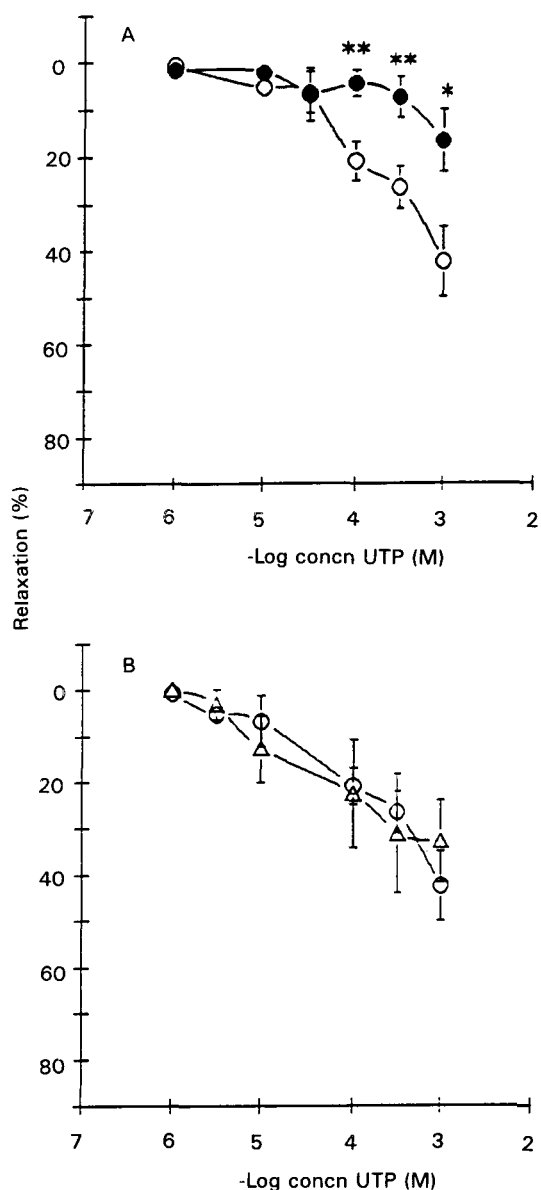


FIG. 3. A. Response to UTP of thoracic aorta in the presence (O) or after removal of endothelium (●). B. Relaxing response to UTP of thoracic aorta, with intact endothelium, in the presence (△) or absence (O) of suramin (0·1 mM). Each point is the mean value ± s.e. from five rabbits. *P < 0·05; **P < 0·01.

with 0·1 mM suramin for 20 min before the cumulative addition of UTP, no difference was detected in the relaxant effect of UTP (Fig. 3B).

### Suramin effect on adenosine-induced relaxation

As reported in Fig. 1D, the relaxant effect of adenosine depends on action directly on smooth muscle. Therefore, the effect of suramin (0·1 mM) on the relaxant effect of adenosine was studied in aortic preparations deprived of endothelium for direct exposure of smooth muscle to the effect of drugs. Suramin did not affect the characteristic relaxant response of adenosine at concentrations ranging from 1 $\mu$M to 1 mM (Fig. 4).

### Effect of $P_1$ antagonists on NECA relaxation

NECA, the stable adenosine analogue that acts as a potent agonist on $P_1$ purinoceptors, produced vascular relaxation that was independent of the presence of endothelium (data not shown), as for adenosine. The effect of NECA was, therefore, investigated in endothelium-deprived aorta preparations. Relaxation was observed at concentrations of 1 $\mu$M– 1 mM and was not affected by the presence of $P_1$ antagonists PACPX (5 $\mu$M) or DMPX (50 $\mu$M), preincubated for 20 min before the cumulative addition of NECA (Fig. 5).

### Potency order of relaxant purine agonists at smooth muscle level

To determine a rank order of potency among various purine agonists at smooth muscle level, the effect of NECA, adenosine and inosine were compared (Fig. 6). Inosine relaxation was not influenced by the presence of endothelium (data not shown), suggesting only a muscular effect, as seen for adenosine. The activity of inosine was similar to that of the muscular component of ATP, ADP and AMP (Fig. 1; for clarity of presentation, the curves of ATP and ADP are not included in Fig. 6). Adenosine and particularly NECA were more potent than inosine (Fig. 6). The rank order of relaxant potency at smooth muscle level is, therefore, NECA > adenosine > ATP $\cong$ ADP $\cong$ AMP $\cong$ inosine.
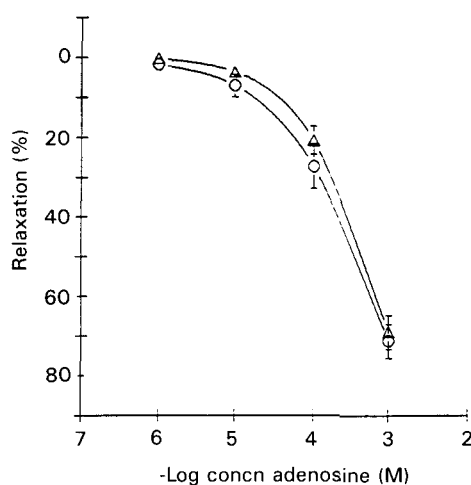


FIG. 4. Relaxing response to adenosine of thoracic aorta, after removal of endothelium, in the presence (△) or absence (O) of suramin (0·1 mM). Each point is the mean value ± s.e. from five rabbits.
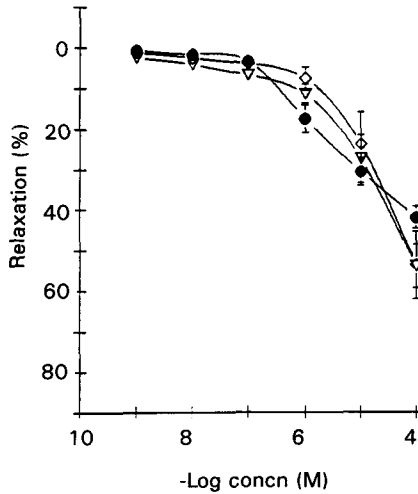
FIG. 5. Response to NECA ot thoracic aorta, without endothelium, in the presence of PACPX ($5 \mu$M) ($\Diamond$) or DMPX ($50 \mu$M) ($\triangledown$). Control without PACPX or DMPX ($\bullet$). Each point is the mean value $\pm$ s.e. from five rabbits.

## Discussion

The present data demonstrate that rabbit aorta relaxation by purines is a phenomenon dependent on $P_{2y}$ purinoceptor and nucleotide receptor, whilst no evidence for $P_1$ purinoceptor is found at the endothelial level. The direct smooth muscle relaxant effect of purines is mediated exclusively by $P_3$ purinoceptor (Fig. 7).

Our findings are consistent with earlier observations that ATP acts as a vascular relaxant at both the endothelial and the smooth muscle level (Furchgott 1984; Chinellato et al 1991). Endothelium-dependent relaxation by ATP was first attributed exclusively to $P_{2y}$ purinoceptors (Burnstock & Kennedy 1985). A new nucleotide (N) receptor has also recently been postulated at the endothelial level, mediating vasodilation by both purines (ATP and ADP (Wilkinson et al 1993)) and pyrimidines (UTP (O'Connor et al 1991; Chinellato et al 1992a; Wilkinson et al 1993)). The present
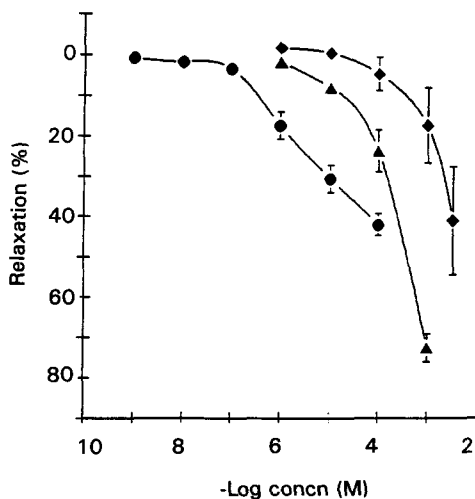
data indicate that, of the purines, ATP and ADP possess endothelial activity, whilst AMP, adenosine and inosine produce no endothelium-dependent vasodilation, but only a muscle-related effect. The endothelial effect of ATP was therefore further investigated in the presence of suramin, that is, a $P_2$-purinoceptor antagonist (Dunn & Blakeley 1988; Hoyle et al 1990), to exclude the effect mediated by the $P_{2y}$-purinoceptor component. In our conditions, endothelium-mediated relaxation by ATP was only partially inhibited by suramin, since ATP relaxation in the absence of endothelium was lower than that in the presence of suramin, in intact endothelium. These data indicate a non-$P_2$-purinoceptor component in the endothelial effect of ATP, agreeing with data obtained in bovine isolated aortic endothelial cells (Wilkinson et al 1993). To characterize this new relaxant effect better, the vascular effect of UTP was also considered. This pyrimidine produced vasodilation by acting mainly at the endothelial level, and relaxation was not antagonized by suramin, showing that this effect is not mediated by any $P_2$ purinoceptors. Moreover, this endothelial relaxant component is not due to $P_1$ purinoceptors, since adenosine did not produce any effect on endothelium. Since UTP was as effective as ATP in producing a relaxant response via this new endothelial site of action, it was considered as a nucleotide (N) pyrimidinoceptor, as suggested by O'Connor et al (1991).

Regarding the muscle relaxant effect of purines, ATP, ADP, AMP, adenosine and inosine were all active. The presence of $P_1$ purinoceptor was tested by means of the potent $P_1$ agonist NECA (Bruns et al 1986) which produced a concentration-dependent vascular relaxation at the smooth muscle level. This effect was excluded as being mediated by $P_1$ purinoceptor because PACPX and DMPX, both potent $P_1$ antagonists (Bruns et al 1983; Seale et al 1988), failed to inhibit NECA relaxation. In aorta preparations deprived of endothelium, suramin was ineffective in inhibiting relaxation mediated by both adenosine and ATP; moreover, as potency in inducing relaxation at the smooth muscle level was 10 times that for adenosine than for ATP, we can exclude any involvement of $P_2$ purinoceptors at the smooth muscle level. We can also exclude muscle relaxation by purines as an N-mediated effect since UTP was ineffective, confirming that a



FIG. 6. Response to NECA ($\bullet$), adenosine ($\blacktriangle$) or inosine ($\blacklozenge$) of thoracic aorta, without endothelium. Each point is the mean value $\pm$ s.e. from five rabbits.
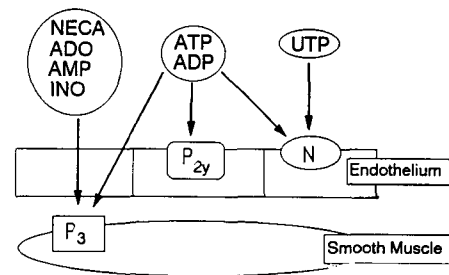


FIG. 7. Schematic drawing of rabbit aorta purinoceptors and pyrimidinoceptor. Adenine nucleotides (ATP, ADP, AMP), adenine nucleosides (adenosine and inosine), and pyrimidine nucleotide (UTP) act at different sites, as indicated. ATP and ADP induce relaxation via $P_{2y}$ purinoceptor, nucleotide pyrimidinoceptor (N), both located at the endothelium level, and via $P_3$ purinoceptor, located in smooth muscle. UTP induces endothelium-dependent relaxation via nucleotide pyrimidinoceptor (N). AMP, adenosine (ADO), inosine (INO) and adenosine analogue (NECA) relax vessel via $P_3$ purinoceptor in smooth muscle.

different class of purinoceptor must be present at the smooth muscle level. This allows us to introduce a further class of purine receptors, $P_3$ purinoceptors, according to our previous preliminary hypothesis (Chinellato et al 1992b); the rank order of potency of the agonists is NECA > adenosine > ATP ≅ ADP ≅ AMP ≅ inosine.

## Acknowledgements

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